

The photosynthetic cytochrome c_{550} from the diatom *Phaeodactylum* *tricornutum*

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Abbreviations:

β -DM, β -dodecyl-maltoside; Cc₅₅₀, cytochrome *c*₅₅₀; Cc₆, cytochrome *c*₆; CW, continuous wave; EPR, electron paramagnetic resonance; HYSCORE, hyperfine sublevel correlation spectroscopy; MALDI-TOF MS, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; PSII, photosystem II.

ABSTRACT

The photosynthetic cytochrome c_{550} from the marine diatom *Phaeodactylum tricorutum* has been purified and characterized. Cytochrome c_{550} is mostly obtained from the soluble cell extract in relatively large amounts. In addition, the protein appeared to be truncated in the last hydrophobic residues of the C-terminus, both in the soluble cytochrome c_{550} and in the protein extracted from the membrane fraction, as deduced by mass spectrometry analysis and the comparison with the gene sequence. Interestingly, it has been described that the C-terminus of cytochrome c_{550} forms a hydrophobic finger involved in the interaction with photosystem II in cyanobacteria. Cytochrome c_{550} was almost absent in solubilized photosystem II complex samples, thus indicating a very low affinity of cytochrome c_{550} for the photosystem II complex. Under iron-limiting conditions the amount of cytochrome c_{550} decreases up to about 45% as compared to iron-replete cells, pointing to an iron-regulated synthesis. Oxidized cytochrome c_{550} has been characterized using continuous wave EPR and pulse techniques, including HYSCORE, and the obtained results have been interpreted in terms of the electrostatic charge distribution in the surroundings of the heme centre.

INTRODUCTION

Photosynthetic cytochrome c_{550} (Cc_{550}) is a c -type heme protein with a very unusual bis-histidinyl axial coordination (Frazão et al. 2001). It is currently accepted that Cc_{550} is an extrinsic protein subunit of photosystem II (PSII), since it appears stoichiometrically bound to the luminal PSII surface in the vicinity of the D1 and CP43 proteins, and close to the oxygen evolving complex (Zouni et al. 2001; Ferreira et al. 2004; Umena et al. 2011; Shen 2015; Ago et al. 2016). Cc_{550} is present in cyanobacteria and in eukaryotic algae from the red photosynthetic lineage, which includes diatoms, but is absent in the green lineage, which comprises green algae and plants, which seem to have replaced Cc_{550} for the non-iron containing PsbP subunit (revised in: Roncel et al. 2012; Ifuku and Noguchi 2016).

The role of Cc_{550} in PSII appears to be stabilizing the Mn_4CaO_5 cluster and the binding of Cl^- and Ca^{2+} ions (Kerfeld and Krogmann 1998; Shen et al. 1998; Bricker et al. 2012). Crystal structures and theoretical calculations suggest that Cc_{550} could also contribute to entry/exit channels for water or protons from the Mn_4CaO_5 cluster (Umena et al. 2011; Vogt et al. 2015), although the role of Cc_{550} has been recently put in discussion (Takaota et al. 2016). Beyond a structural function, a redox role of the cytochrome heme cofactor in PSII has not been established. In addition, in many organisms Cc_{550} can be mostly purified as a soluble protein (Evans and Krogmann 1983; Navarro et al. 1995; Kerfeld and Krogmann, 1998). Thus, it would be possible that two different populations of Cc_{550} are present: one bound to the PSII and the second one soluble in the lumen. Several roles for this "extra" free Cc_{550} have been proposed in cyanobacteria, mostly in anaerobic carbon and hydrogen metabolism (Krogmann 1991; Morand et al. 1994; Kang et al. 1994), cyclic photophosphorylation (Kienzel and Peschek 1983) and in the reduction of nitrate to ammonia (Alam et al. 1984).

Cyanobacterial Cc_{550} shows intriguing structural and biophysical properties. In addition to the unusual bis-histidinyl axial heme coordination, the protein has a very low midpoint redox potential (E_m) when purified as the soluble form (from -250 to -314 mV) (Alam et al. 1984; Navarro et al. 1995; Roncel et al. 2003), but much more positive potential values were obtained for the Cc_{550} bound to PSII (from -80 to $+200$ mV) (Roncel et al. 2003; Guerrero et al. 2011). This latter fact allowed proposing a possible role for Cc_{550} as an electron donor to the Mn_4CaO_5 cluster in a photoprotective cycle (Guerrero et al. 2011). On the other hand, the EPR spectra of the different cyanobacterial Cc_{550} studied in the oxidized

form are typical of a low-spin heme with bis-histidine coordination (Roncel et al. 2003; Kerfeld et al. 2003). Finally, minor, but significant differences in the EPR spectra from the free and PSII-bound Cc₅₅₀ were observed (Roncel et al. 2003; Kirilovsky et al. 2004).

Diatoms belong to the red lineage of algae that diverged along evolution from the green lineage that evolved to higher plants (Bowler et al. 2008; Grouneva et al. 2013) and nowadays constitute the most abundant and diversified group of oceanic eukaryotic phytoplankton (Kooistra et al. 2007; Bowler et al. 2010). The photosynthetic chain in diatoms possesses some peculiarities, arising from their double endosymbiotic origin. Thus, the assembly of extrinsic proteins at the luminal side of PSII includes the three cyanobacterial-like subunits PsbO, PsbU and PsbV (or Cc₅₅₀), as well as the PsbQ' subunit also present in red algae (Enami et al. 1998; Okumura et al. 2008; Nagao et al. 2010). However, besides these subunits, diatoms have an extra extrinsic protein, named as Psb31 (Okumura et al. 2008; Nagao et al. 2010). Reconstitution experiments of isolated PSII samples depleted of the extrinsic subunits indicate that both in red and diatoms algae the binding of PsbV/Cc₅₅₀ requires prior binding of PsbO and PsbQ' and, in the case of diatoms, of Psb31 (Enami et al. 1998, 2003; Nagao et al. 2010). This contrasts with the results obtained in cyanobacteria, where Cc₅₅₀ is able to bind directly to the PSII core complex in a manner essentially independent of other extrinsic subunits (Enami et al. 2003). It is interesting, however, to note that the very recent crystal structure of the PSII from the red alga *Cyanidium caldarium* has shown an overall structure similar to the cyanobacterial complex, including the position of Cc₅₅₀ in PSII (Ago et al. 2016).

In this work, we have purified and characterized the Cc₅₅₀ from the diatom *Phaeodactylum tricorutum*. The protein is obtained in a truncated C-terminal form with a low affinity for the PSII complex. In addition, the characterization of *P. tricorutum* Cc₅₅₀ by continuous wave and pulse EPR indicates a relationship between the electrostatic environment of the heme centre within the protein heme-pocket and the electronic structure of the paramagnetic entity.

EXPERIMENTAL PROCEDURES

Cell cultures

Cells from the coastal diatom *Phaeodactylum tricornutum* CCAP 1055/1 (hereafter *Phaeodactylum*) were used as biological material. *Phaeodactylum* cells from photobioreactors outdoor cultures were obtained as a frozen paste from Easy Algae (Cádiz, Spain). Alternatively, *Phaeodactylum* was grown in Artificial Seawater (ASW) medium (McLachlan 1964; Goldman and McCarthy 1978) in a rotatory shaker (50 rpm) at 20 °C. The cultures were illuminated by fluorescent white lamps giving an intensity of 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ under a light/dark cycle of 16/8 h. For the experiments of the effects of iron deficiency, cells from cultures of 15 days were pelleted at 5,000xg for 5 min and grown in standard ASW medium (iron-replete culture; 12 $\mu\text{M Fe}$) and ASW medium with only 0.12 $\mu\text{M Fe}$ (iron-deplete culture), with regular transfer of the cells into fresh media. In the experiments of Cc_{550} and cytochrome c_6 (Cc_6) quantification after changing iron availability, cultures grown under iron-replete or iron-deficiency were divided in two equal volumes, centrifuged (5,000xg for 5 min) and resuspended in the same volume of iron-replete or iron-deficient media. Four sets of samples were thus obtained: (1) cells growing in iron-replete medium and (2) cells growing in iron-deficient medium, resuspended in the same fresh medium; (3) cells growing in iron-deficient medium resuspended in fresh iron-replete medium; and (4) cells growing in iron-replete medium resuspended in fresh iron-deficient medium.

Proteins purification

Purification of Cc_{550} from *Phaeodactylum* cells was carried out as a modification of the procedure recently described for the purification of Cc_6 from the same organism (Navarro et al. 2011; Bernal-Bayard et al. 2013). The method consisted of cell resuspension in 10 mM MES, pH 6.5, 2 mM KCl and 5 mM EDTA buffer, supplemented with DNase and the protease inhibitors PMSF, benzamidine, aminocaproic acid and a tablet of the cComplete Protease Inhibitor Cocktail (Roche), followed by French press disruption (20,000 psi), treatment with streptomycin sulfate, sequential precipitation with 30 and 60 % ammonium sulfate and extensive dialysis, to obtain the clarified crude extract. From this point Cc_{550} was purified by FPLC, first by using a DEAE Sepharose column (Cc_{550} elution by applying a 0.01–0.2 M NaCl linear gradient in Tris-HCl 10 mM, pH 7.5 buffer) and further by gel filtration using a Sephacryl S-200 HR column (GE Healthcare Life Sciences). Protein fractions with an A_{550}/A_{275} ratio close to 1.0 were pooled, suspended in Tris-HCl 10 mM, pH

7.5 buffer, concentrated in an Amicon pressure filtration cell, and finally frozen at $-80\text{ }^{\circ}\text{C}$ until use. The concentration of Cc_{550} was calculated using an extinction coefficient of $26\text{ mM}^{-1}\text{ cm}^{-1}$ at 550 nm for the reduced form (Shimazaki et al. 1978; Navarro et al. 1995).

PSII-enriched samples from *Phaeodactylum* cells were obtained by β -dodecyl-maltoside (β -DM) solubilization and sucrose gradient separation. Fresh *Phaeodactylum* cells were resuspended in 50 mM MES, pH 6.5, 5 mM MgCl_2 and 5 mM EDTA buffer (buffer A), supplemented with proteases inhibitors and 1 M betaine or sorbitol (buffer B), and disrupted in a French pressure cell at 7,000 psi. Some control experiments were carried out with cells resuspended in buffer A (non-osmotically stabilized buffer) and disrupted by 6 cycles of freezing in liquid nitrogen and thawing at $25\text{ }^{\circ}\text{C}$ in a thermoblock. In any case, unbroken cells were separated by centrifugation at $5,000\times g$ for 5 min and the supernatant (crude extract) was centrifuged at $170,000\times g$ for 30 min. The resultant supernatant was considered as the soluble fraction, whereas the pellets were resuspended in buffer B and centrifuged as before to obtain a washed fraction (the supernatant) and a thylakoids extract (the pellets). Pellets were resuspended in buffer A supplemented with 0.2 M sucrose at 1 mg Chl mL^{-1} and later diluted to $0.5\text{ mg Chl mL}^{-1}$ with the same volume of β -DM 3% (w/v), prepared in buffer A, to yield a final detergent:Chl ratio of 30:1 (w/w). After 30 min in the dark at $4\text{ }^{\circ}\text{C}$ under gentle stirring, the solution was centrifuged at $170,000\times g$ for 30 min. The resulting supernatant (detergent-solubilized fraction) was loaded onto a continuous sucrose density gradient from 0.17 to 0.47 M sucrose, prepared in buffer A + 0.03% β -DM, and centrifuged at $135,000\times g$ for 16 h. The medium mostly-green band was collected and considered as a PSII-enriched sample. The PSII content was calculated from the differential absorbance change of the PSII-intrinsic cytochrome b_{559} protein (ascorbate minus ferricyanide) (Roncel et al. 2003). The content of Cc_{550} was estimated from the absorbance difference at 550 nm between the reduced (sodium dithionite, 1 mM) and oxidized state (in the presence of sodium ascorbate 1 mM), using a differential extinction coefficient (reduced minus oxidized) of $15\text{ mM}^{-1}\text{ cm}^{-1}$ at 550 nm (Navarro et al. 1995).

Analytical methods

The N-terminus of purified Cc_{550} was sequenced in a Procise TM 494 Protein Sequencer (Applied Biosystems) at the *Protein Chemistry Service* (CIB-CSIC, Spain). Redox titrations were performed as described previously (Molina-Heredia et al. 1998; Guerrero et al. 2014) in potassium phosphate 50 mM (pH 7) or acetic-acid/MES (25:25 mM, pH 5-6) buffers, in the presence of $10\text{ }\mu\text{M}$ of anthraquinone-2-sulfonate, 2-hydroxy-1,4-naphthoquinone and

duroquinone as redox mediators. The accuracy of the potential-measuring system was first tested by redox titration of a flavin-mononucleotide solution as a standard ($E_{m,7} = -220$ mV). Chlorophyll concentrations were determined as previously reported (Arnon 1949; Jeffrey and Humphrey 1975).

The total Cc₅₅₀ content in *Phaeodactylum* cells was determined by differential absorbance measurements. 40-100 mL cultures were precipitated by centrifugation at 16,000xg for 5 min and wet pellets were weighed. Cells were then resuspended to 1 mL in culture media and frozen until use. Unfrozen samples were disrupted by 6-7 cycles of freezing in liquid nitrogen and thawing at 30 °C in a thermoblock. Soluble fractions were obtained by centrifugation at 16,000xg for 15 min, and the content of Cc₅₅₀ was estimated as before. This method extracted up to 85-90% of Cc₅₅₀, as determined by further protein extraction by sonication of the membrane fractions. Control measurements of the Cc₆ content were made from the absorbance difference at 552 nm between the fully reduced (sodium ascorbate, 1 mM) and fully oxidized (potassium ferricyanide, 0.5 mM) states (Roncel et al. 2016). The amount of Cc₅₅₀ or Cc₆ was related to grams of the initial wet weight. Some additional experiments were designed to estimate the amount of soluble (or easily removed from membranes) and membrane-associated Cc₅₅₀ (and Cc₆). Briefly, *Phaeodactylum* cells were resuspended in 50 mM MES, pH 6.5, buffer supplemented with 10 mM MgCl₂, 1 M betaine, proteases inhibitors and DNase, and disrupted by a French press cycle at 7,000 psi. Unbroken cells were separated by centrifugation at 5,000xg for 5 min and the supernatant was centrifuged at 170,000xg for 25 min. The resultant supernatant was considered as the soluble fraction, whereas the pellet was resuspended in the same buffer and centrifuged as before to obtain a washed fraction (the supernatant) and a membrane extract (the pellet). Cc₅₅₀ was extracted from this membrane fraction by resuspension in 50 mM MES buffer, pH 6.5, supplemented with 500 mM NaCl and 4 % Triton X-100 detergent, followed by 30 min incubation in the dark. Solubilized proteins were separated by centrifugation (170,000xg for 25 min) and partially purified by sequential precipitation with 50 and 85 % ammonium sulfate. The final pellet (membrane associated fraction) was resuspended in few mL of potassium phosphate 50 mM, pH 7, buffer, and the amounts of Cc₅₅₀ (and Cc₆) were estimated as before. Alternatively, the final pellet was resuspended in pure water, washed by two dilution/concentration cycles in an Amicon pressure filtration cell, and used for molecular weight MALDI-TOF analysis.

For the immunodetection of Cc₅₅₀, polyclonal antibodies raised against this cytochrome were generated using standard procedures at the *Animal Experimentation*

Facility (University of Seville, Spain) by subcutaneous injection of 1 mg of purified protein into a white New Zealand rabbit (Bernal-Bayard et al. 2013). Antibodies against D1 (Agriserá, Sweden) and Psb31 from the diatom *Chaetoceros gracilis* (a generous gift of Prof. T. Tomo, Tokyo University of Science, Japan) were also used. Protein samples or cell extracts were resolved on 15% (w/v) polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Protran Premium 0.45 μm NC, GE Healthcare Life Sciences). The membrane was incubated overnight with the primary antibodies (dilution 1:1,000) followed by 1 h incubation with Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (Biorad) (dilution 1:10,000), and visualized with the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS analyses were performed at the *Proteomic Service* (IBVF, Sevilla, Spain), in an Autoflex model analyzer (Bruker Daltonics, Germany) operated in lineal (protein molecular weight) or reflector (peptide mass fingerprint) positive modes. Mass spectra were previously calibrated with appropriate standards to the range of mass under study. The molecular weight (MW) of C_{550} was determined with sinapinic acid as matrix, whereas HCCA (α -cyano-4-hydroxy-cinnamic acid) was used as the matrix for peptide mass fingerprint. Tryptic digestion and BrCN cleavage were carried out as described elsewhere (Sechi and Chait 1998; Crimmins et al. 2005; Martínez-Fábregas et al. 2014) and the peptide fingerprint was obtained by MALDI-TOF MS. Protein identification was carried out by comparing the obtained peptide fingerprint with the NCBI database using the MASCOT software programs.

Electron paramagnetic resonance (EPR) spectra

Protein samples for EPR were prepared in Tris-HCl 10 mM, pH 7.5 buffer, supplemented with glycerol in a 2:1 ratio, to obtain a glass upon freezing. Subsequently, samples were transferred to 4 mm quartz EPR tubes, frozen in liquid nitrogen and stored until use. The resulting protein concentration was 0.6 mM.

Both, continuous wave (CW) and pulse EPR measurements were performed on a Bruker Eleksys spectrometer (Bruker Biospin, Germany) operating at X-Band (about 9.6 GHz), either equipped with a rectangular cavity operating in the TE₁₀₂ mode or a DM5 dielectric ring resonator, for CW and pulse measurements respectively. The experiments

were performed at very low temperatures by means of a helium gas-flow cryostat and a temperature controller, both from Oxford Instruments (UK).

The CW-EPR spectra were taken at 25 K adjusting the microwave power to ensure that there was no saturation. Modulation frequency and amplitude of the magnetic field were 100 Hz and 1 mT respectively. All pulse EPR experiments were recorded between 6 and 8.5 K and a shot repetition time of 2 ms. Electron Spin Echo detected field-sweep spectra were recorded with the Hahn-echo sequence $\pi/2 - \tau - \pi$. 2D Hyperfine Sublevel Correlation experiments (HYSCORE) were performed using the standard sequence $\pi/2 - \tau - \pi/2 - T1 - \pi - T2 - \pi/2$ with an eight-step phase cycle (Schweiger and Jeschke 2001).

Processing of the 2D HYSCORE spectra included a polynomial baseline correction, hamming windowing in both dimensions before performing a 2D Fast Fourier Transform. The absolute value of this transform was displayed in the 2D frequency domain.

Structural model

The structure of Cc₅₅₀ from *Phaeodactylum* was modeled using the program Phyre² (<http://www.sbg.bio.ic.ac.uk/phyre2/html/>) (Kelly and Sternberg 2009), employing as main templates the crystal structures of Cc₅₅₀ from *Thermosynechococcus elongatus* (pdb 1MZ4 and 1W5C) and *Synechocystis* sp. PCC 6803 (pdb 1E29). Surface electrostatic potentials were calculated and represented using the Swiss-Pdb Viewer Program (Guex and Peitsch 1997).

RESULTS

Protein purification and analytical characterization

Figure 1 shows the different purification and protein extraction procedures here carried out during the characterization of Cc₅₅₀ from the marine diatom *P. tricornutum*. First, by following a modification of previously described purification methods (see the Experimental Procedures section), a yield of ca. 15 mg of purified Cc₅₅₀ was obtained from 100 g wet weight of *Phaeodactylum* cells, from about 30 mg present in the initial supernatant after the streptomycin sulfate treatment, as determined by the differential absorbance changes (not shown). The Cc₅₅₀ obtained in the soluble fraction was about 85% of the total (*i.e.*, 35 mg), as also estimated from differential absorbance changes. Thus, *Phaeodactylum* cells disruption at high pressure in a non-osmotically stabilized medium allowed to extract moderately large amounts of solubilized Cc₅₅₀. Visible absorption spectra of purified Cc₅₅₀, both in the native oxidized and dithionite-reduced forms, show absorption bands (549.5, 521 and 417 nm, reduced; 405.5 and 528.5 nm, oxidized) similar to those previously described (Shimazaki et al. 1978; Navarro et al. 1995) (Figure S1, supplementary material). The absorbance ratio A_{275} (oxidized)/ A_{550} (reduced) for the final purest protein samples was 1.07. Redox titration of *Phaeodactylum* Cc₅₅₀ established a midpoint redox potential ($E_{m,7}$) value of -190 ± 12 mV (Figure S1, supplementary section) which did not significantly change in the pH range 5-7 (data not shown). This potential value, although maintaining the typical negative redox potential, is significantly more positive than those described in cyanobacteria for Cc₅₅₀ in solution (-250 to -300 mV) (Navarro et al. 1995; Roncel et al. 2003; Guerrero et al. 2011).

Interestingly, when checking the MW of purified Cc₅₅₀ by MALDI-TOF analysis, a value of ca. 15,110 Da was obtained (Figure 2A). After subtracting the heme group (616 Da), a MW of ca. 14,495 for the peptide chain is consequently deduced. This value is lower than the theoretical value inferred from the *psbV* gene sequence (ca. 14,822 for the peptide chain and 15,438 for the heme-containing holoprotein; see Figure 2) but agrees with a truncated protein in the two last tyrosine residues of the C-terminus (14,495.5 for the peptide chain and ca. 15,111 for the holoprotein; Figure 2). Actually, no signal corresponding to the theoretical sequence has been detected in any case, although an even smaller band of much lower intensity was also identified, whose MW (14,997.6 Da) could fit with an additional small fraction of a truncated protein in the last three residues of the C-terminus (14,998.4 for the peptide chain; Figure 2).

Different experiments were carried out to confirm the occurrence of a C-terminal truncated protein. First, the N-terminal part of purified Cc₅₅₀ was sequenced (data not shown), showing the correct sequence according to the *psbV* gene (IDLDEATRTV; Figure 2, lower). Second, Cc₅₅₀ samples were subjected to trypsin or BrCN cleavage and peptide analysis (Figure S2, supplementary material). Trypsine digestion unequivocally identified the sample as the Cc₅₅₀ protein, without the observation of additional peptides arising from alternative proteins (data not shown). However, lysines 129 and 134 in Cc₅₅₀ (targets for trypsin) prevented the possible identification of the last protein C-terminus part when using this protease, and thus BrCN was alternatively used. As shown in Figure S2 (supplementary material), BrCN cleavage allowed the identification of peptides covering residues 1-115, but the expected peptide corresponding to the 116-137 residues in the C-terminus (MW=2,471.9 Da) was absent. Conversely, new peptides compatible with the lack of the 2-3 C-terminus groups appear (Figure S2), thus confirming the occurrence of truncated species. It is also important to note that the truncated Cc₅₅₀ not only appears in the final purified protein, but also in intermediate steps of the purification process (data not shown).

In order to better establish the Cc₅₅₀ distribution and nature (truncated or not) between soluble (or easily membrane-released) and membrane-associated fractions, *Phaeodactylum* cells were disrupted in osmotically stabilized media (in the presence of betaine or sorbitol) under a lower pressure (7,000 psi) and Cc₅₅₀ was quantified by the differential absorbance changes, both in the soluble fraction and in the fraction extracted from membrane samples treated with 500 mM NaCl and 4% Triton X-100 (Figure 1). As an additional control, the soluble luminal Cc₆ protein was also quantified in the different samples. From the differential absorbance spectra (reduced *minus* oxidized) corresponding to Cc₅₅₀ and Cc₆ in samples obtained after treating the membrane fractions with NaCl and detergent (Figure S3, supplementary material), it was possible to estimate that the membrane-associated proteins stand for \approx 40% and 10% of total Cc₅₅₀ and Cc₆, respectively (60% and 90% in the soluble protein fraction), no differences being observed when using betaine or sorbitol as osmotic stabilizing agents (data not shown). The presence of a small amount of Cc₆ in the membrane-extracted fraction, as well as the fact that washing the membranes with the disruption buffer, instead the salt/detergent mixture, did not result in a significant extraction of either Cc₅₅₀ or Cc₆ (not shown), indicate that at least a part of the membrane-extracted Cc₅₅₀ would arise from disruption of closed thylakoids during the detergent washing procedure. To test if the soluble or the membrane-associated Cc₅₅₀ correspond to different forms (*i.e.*, a soluble but truncated protein, and a membrane-bound

and complete protein) a MW MALDI-TOF analysis of Cc₅₅₀ partially purified from the membrane-extracted fraction was carried out. The results, although more uncertain due to the lower protein concentration and to interferences from other membrane components, indicated again a truncated protein, similar (MW \approx 15,106) to that obtained in the soluble fraction, without any detection of the theoretical complete protein (Figure 2B).

The affinity and association of Cc₅₅₀ to PSII has been investigated by Western blot analysis of the different fractions acquired along the obtention of PSII-enriched samples from *Phaeodactylum* by β -DM solubilization (Figure 1), a standard method used for PSII purification (Enami et al. 1995; Bumba et al. 2004; Kirilovsky et al. 2004; Nagao et al. 2007). Cc₅₅₀ and both the D1 core and the extrinsic Psb31 subunits of PSII were monitored. Psb31 is exclusive of diatoms, and its presence along PSII purification is particularly relevant as it has been described to be required for the binding of Cc₅₅₀ to the photosystem complex (Okumura et al. 2008; Nagao et al. 2010). Additionally, direct spectroscopic monitoring of the PSII core (the cytochrome *b*₅₅₉) and Cc₅₅₀ in the different fractions from the sucrose gradient was also carried out. From the immunological analysis shown in Figure 3 it is first confirmed that although Cc₅₅₀ mostly appears in the soluble fraction, a significant amount of the protein can be observed both in the initial and washed membrane fractions, as well as in the β -DM solubilized sample, together with the D1 and Psb31 subunits (Figure 3, *upper*). However, after sucrose gradient partitioning, Cc₅₅₀ is located mostly in the top low-density fraction, corresponding to free (not-associated to PSII) Cc₅₅₀ (Figure 3, *upper*), whereas D1 and Psb31 only appear in the high-density lower green band, containing PSII. The low content of Cc₅₅₀ in the PSII-enriched samples was also confirmed by spectroscopic measurements of the differential absorbance changes associated both to this protein and cytochrome *b*₅₅₉. Thus, whereas cytochrome *b*₅₅₉ was clearly monitored, only minor changes at 562 nm (probably associated with the cytochrome *b*_{6f} complex) were observed under dithionite reduction, and no significant changes associated to Cc₅₅₀ were detected (Figure 3, *lower*), thus over confirming the very low affinity of *Phaeodactylum* Cc₅₅₀ for the PSII complex.

It is well known that iron availability limits growth of photosynthetic algae and of diatoms in particular (Allen et al. 2008; Morrissey and Bowler 2012; Nunn et al. 2013). A down-regulation under iron limitation of several iron-containing proteins has been previously reported in *Phaeodactylum*, although the global PSII concentration and D1 transcription is maintained, and other PSII subunits, including Cc₅₅₀, were described to remain almost constant (Allen et al. 2008). Recently we have reported a decrease to a level

of ca. 30% of the Cc_6 protein content in iron-deplete cells as compared with iron-replete conditions (Roncel et al. 2016). Considering that at the protein level the amount of both Cc_6 and Cc_{550} is similar in *Phaeodactylum* cells (this work, and see Bernal et al. 2013; Roncel et al. 2016), we have here investigated the evolution of the Cc_{550} content when changing iron availability. It is interesting first to note that cultures grown under low iron availability showed levels of Cc_6 (not shown) and Cc_{550} of 25-30% and 45-50%, respectively, compared with iron-replete conditions, as estimated by its specific redox differential absorbance changes (Figure 4A). Thus, from these values it seems that down-regulation under low iron of the electron donor to PSI (the Cc_6) is higher than the PSII-associated Cc_{550} protein. In addition, cultures grown under iron-replete or iron-limiting conditions were collected and resuspended in the same volume of iron-deplete or iron-replete media, respectively, and the content in Cc_{550} (and Cc_6) was followed during several days of culture. As shown in Figure 4B, when shifting from replete to deplete (+/-) or from deplete to replete (-/+) conditions, a decrease or a parallel increase in the content of Cc_{550} , respectively, were observed, these changes occurring during the first 6 days of culture. Similar qualitative results were obtained when analyzing the Cc_6 content (not shown).

EPR measurements

The CW-EPR spectrum of the soluble form of *Phaeodactylum* Cc_{550} is presented in Figure 5A, where it shows the three characteristic features of a low-spin heme ($S = 1/2$), with g factor absolute values of $|g_z| = 3.00$, $|g_y| = 2.24$ and a broad signal at high field centered at $|g_x| = 1.44$ (Table 1). The electron spin echo (ESE) detected EPR spectrum, normally much more sensitive to broad signals since it is displayed in the absorption mode, confirms the g values (Figure 5B). EPR spectra of low-spin heme centers are usually analyzed with the *hole-model* (Griffith 1957; Taylor 1977). Using this model it is possible to obtain the relative energy levels of the t_{2g} orbitals of the iron atom, where the unpaired electron is distributed (Alonso et al. 2007; Alonso and Martínez 2015). The level distribution can be parametrized by the crystal field parameters Δ and V (Figure 5C), which can be calculated in units of the spin-orbit coupling constant, λ , from the g -values. In our case, the estimated values (Table 1) were: $\Delta/\lambda = 3.17$, $V/\lambda = 1.71$, and subsequently $V/\Delta = 0.54$. These parameters are typical for a bis-histidine coordination (Peisach et al. 1973, and see Table 1). HYSCORE experiments were undertaken in this variant to study the hyperfine interaction of the electron spin in the iron with the nuclear spin ($I=1$) of the coordinating nitrogens (Figure 5D). The experiments

were performed at the magnetic field corresponding to g_z ($B = 230$ mT), where the magnetic field is perpendicular to the heme plane. In the negative quadrant of the experiment, it can be observed the so-called double-quantum (dq) correlation peaks (Figure 5D), which are the ones normally more intense in HYSCORE spectra of low-spin heme proteins (García-Rubio et al. 2003; Ioanimescu et al. 2007). In this case, and unlike other proteins and low-spin heme model complexes where one peak for heme and one peak for histidine are observed at this position, up to four such peaks are solved. The assignment of these peaks to particular nitrogens is difficult, due to the low sensitivity in the single-quantum region at lower frequencies. Irrespectively of the particular assignment of peaks in the spectrum to coordinated nitrogen atoms, there is certainly a lack of equivalency in the hyperfine coupling of the heme nitrogens, since at least two of the peaks have to be assigned to heme nuclei (there are four peaks and four heme nitrogens and two histidine nitrogen nuclei). Similar inequivalencies of heme nitrogens in HYSCORE spectra have already been reported in other heme proteins (Van Doorslaer et al. 2012).

DISCUSSION

Cc₅₅₀ is an extrinsic component in the luminal side of PSII in cyanobacteria, but also in eukaryotic algae from the red photosynthetic branch, which comprises diatoms (Roncel et al. 2012; Ifuku and Noguchi 2016). However, Cc₅₅₀ is missing in the green lineage, which includes green algae and plants, which have replaced this heme-protein for other PSII subunits lacking redox groups (reviewed in Ifuku and Noguchi 2016). Taking into account the peculiar evolution of diatoms, we have here characterized the Cc₅₅₀ from the model diatom *P. tricornutum*, in order to shed light on the different evolutionary pathways of PSII in the different branches of photosynthetic organisms.

Cc₅₅₀ is mainly obtained from the soluble cell extract in relatively large amounts. A standard midpoint redox potential, $E_{m,7}$, value of ca. -190 mV was estimated for the purified protein. This value is at least 60 mV more positive than values described in cyanobacteria at pH 7 for the protein in solution (Navarro et al. 1995; Roncel et al. 2003; Guerrero et al. 2011). Although in *T. elongatus* the redox potential is pH-dependent and varies from -150 to -350 mV as the pH increases from 5 to 10 (Roncel et al. 2003), in *Phaeodactylum* the redox potential remains basically constant in the pH range from 5 to 7. Remarkably, more positive but pH-independent redox potential values (varying from -80 to $+200$ mV) have been obtained for the Cc₅₅₀ bound to PSII (Roncel et al. 2003; Guerrero et al. 2011).

It is interesting to compare the Cc₅₅₀ content in *Phaeodactylum* cells (ca. 35 mg from 100 g of wet weight) with the luminal (and soluble) Cc₆ (ca. 25 mg in the same cell amount), which corresponds to a protein concentration Cc₆/Cc₅₅₀ ratio of ≈ 1.15 . It has been previously reported that the Cc₆ concentration in the thylakoid lumen would be as high as ca. 200 μ M (Haehnel et al. 1989; Durán et al. 2005), which is in agreement with our protein content measurements in *Phaeodactylum* cells. Thus, according to this comparison, a concentration of Cc₅₅₀ in the lumen of ca. 175 μ M could be estimated.

Phaeodactylum Cc₅₅₀ is purified in a truncated form, lacking the last two C-terminal tyrosines, as clearly demonstrated by MS analysis (Figure 2), although a much smaller population of a truncated form lacking the last three C-terminal residues cannot be discarded. Thus the question arises about the physiological relevance of this fact, *i.e.*, if the truncated Cc₅₅₀ is the result of a specific processing or to the unspecific exposition of the protein to cell proteases during the purification course. Although the occurrence of an artefactual protein truncation cannot be totally rejected, several facts speak in favor of a physiological process. First, the purification procedure has been carried out in the presence

of a wide battery of proteases inhibitors. Second, in spite of the relatively high amount of Cc₅₅₀ present in the initial crude extracts (see above), no traces of the theoretical complete protein have been detected in any case during the different steps of purification. Finally, the analysis of Cc₅₅₀ extracted from membrane fractions also points to a physiological truncated protein form. The procedure followed in these latter experiments (lower pressure disruption, membranes washing and detergent extraction) yielded a substantial membrane-bound Cc₅₅₀ population, even partially arising from a small but significant thylakoid fraction enclosing the protein, as deduced by the presence of detectable amounts of the luminal soluble Cc₆. A membrane-extracted Cc₅₅₀ should not have been in contact with other proteases than those from the chloroplast, since the Cc₅₅₀ bound to PSII would have its C-terminus not accessible to proteases, as deduced by the known PSII crystal structures of cyanobacteria and red algae (Shen 2015; Ago et al. 2016, and see below). It is interesting to note that in the diatom *Thalassiosira oceanica*, in addition to the canonical Cc₅₅₀ gene with a KIYF C-terminus sequence, an additional Cc₅₅₀-like gene (ca. 97 % identity) corresponding to a protein with a truncated C-terminus sequence, lacking the three last hydrophobic residues, has been reported (THAOC_28383 gene).

If the processing of Cc₅₅₀ is a specific physiologically relevant event, it could occur either at the RNA or the protein level, in this latter case probably associated to a carboxypeptidase activity. Several serine and zinc carboxypeptidases are annotated in the *Phaeodactylum* genome, although a chloroplast location is not established (Bowler et al. 2008). Thylakoid proteolytic activities are mainly associated to PSII turnover, related to photochemical oxidative effects and to dynamic adaptations under different environmental conditions, as can be high light (Aro et al. 1993; Kato and Sakamoto 2010). An enhanced PSII turnover has been suggested in diatoms (Lavaud et al. 2016), and in *C. gracilis*, in particular, the PSII complex was described to be remarkably unstable and rapid protein degradation was observed (Nagao et al. 2007, 2012). In addition, at least four new proteases were detected in the thylakoid membranes of this diatom (Nagao et al. 2012). It is interesting to note that from the first crystal structure of soluble Cc₅₅₀ from the cyanobacterium *Synechocystis* sp. PCC 6803, it was initially suggested that residues of the C-terminal form a hydrophobic finger maybe involved in the interaction with PSII (Figure 6) (Frazao et al. 2001). This proposal has been later confirmed in the structure of PSII from the cyanobacterium *T. elongatus* (Shen 2015) and, very recently, in the PSII structure from the red alga *C. caldarium* (Ago et al. 2016). The distribution of charges on the surface of the Cc₅₅₀ supports the hypothesis that binding to PSII could be due to electrostatic and

hydrophobic interactions (Guerrero et al. 2011). Furthermore, in *T. elongatus* the last residues in the C-terminus of the Cc₅₅₀ are not resolved in the soluble structure but are visible in the crystal structure, when Cc₅₅₀ is bound to PSII (Kerfeld et al. 2003). This indicates that this region is much more flexible when the cytochrome is in its soluble form, pointing to a direct role in binding to PSII, where this region of the protein is structured. Thus it is possible to speculate that a truncated protein in its C-terminus could have a diminished affinity for the PSII complex and thus a facilitated release during PSII turnover. The modelled structure of *Phaeodactylum* Cc₅₅₀ displays a general folding very similar to that described in other cyanobacterial and red algae Cc₅₅₀ (Figure 6), and thus the complete diatom protein shows the hydrophobic protuberance pointing up –according the orientation presented in Figure 6–, although this protuberance is sensibly diminished in the truncated Cc₅₅₀ form. Interestingly, the electrostatic surface of the diatom cytochrome also shows a distinctive character, as the protein exhibits a diminished negatively charged surface (Figure 6). This fact would be also relevant in setting the affinity binding to PSII.

From the *g*-values obtained from the EPR spectra, it is possible to calculate the crystal field parameters Δ/λ and V/λ and reconstruct the energy levels of the t_{2g} orbitals (Alonso et al. 2007). There have been quite a lot of very informative studies on bis-imidazole model complexes to determine how the geometry of the axial ligands can affect this energy diagram, interpreting it in terms of π donation, steric hindrance or other kinds of interactions (Walker et al. 1986; Quinn et al. 1987). In such studies, the crystal field parameters, and especially *V*, are linked with the dihedral angle between histidines (Walker et al. 1986). When the imidazole planes of the two axial ligands are parallel to each other, a relationship between the angle of the imidazole planes and the axis $N_p - Fe - N_p$ is found (Quinn et al. 1987). Based on these studies on heme model complexes, the parameter V/Δ for the cyanobacterial *T. elongatus*, *Synechocystis* 6803 and *Arthrospira maxima* Cc₅₅₀ hemes was related to a figure accounting for the “global distortion” of the axial ligands, quantified as the sum of a total of eight angles obtained from the three crystalline structures known at the time of this study (Kerfeld et al. 2003). According to this analysis, an important further distortion of the axial ligands upon binding of Cc₅₅₀ to PSII should be expected. However, at present the published structures of PSII from the cyanobacterium *T. elongatus* and the red alga *C. caldarium* (Loll et al. 2005; Ago et al. 2016) show only a very minor rotation of the heme upon binding, as compared with soluble Cc₅₅₀. Moreover, changes in the geometry of the axial ligands among the different soluble variants are also

very moderate, and they do not correspond with the results of Quinn et al. (1987), where a decrease in V/Δ from 0.62 to 0.54 corresponds to a rotation of both imidazole planes of around 20 degrees. Therefore, the observed differences in g -values among the different soluble Cc₅₅₀ variants cannot, or at least not entirely, be due to the very minor observed differences in the axial ligand geometry (Frazao et al. 2001; Kerfeld et al. 2003).

The g -values are also known to be very sensitive to changes in the electrostatic environment of the paramagnetic centre and, related to it, to hydrophobicity changes (Yruela et al. 2003). This sort of changes does not exist in single crystals of model compounds, but could play a very important role in the different Cc₅₅₀ variants. Although the backbone structure is highly conserved among different Cc₅₅₀ proteins, important differences in polarity and surface charge distribution exist (Figure 7). Note that non-conserved residues close to both His67 and His118 axial ligands (*i.e.* A65Q, G69Q, I114Y and A115S in the alignment shown in Figure S4, supplementary data) change the polarity in the heme pocket of *Synechocystis* Cc₅₅₀ respect to *T. elongatus*, which is much closer to *Phaeodactylum* (Figure 7). The variations observed in the g -values and related crystal-field parameters (Table 1) could be associated to these polarity changes. Considering again that binding to PSII will probably involve electrostatic and hydrophobic interactions not far away from the heme (Guerrero et al. 2011; Shen 2015; Ago et al. 2016), it could be responsible for the changes observed in g -values between soluble and PSII-bound Cc₅₅₀ variants. In turn, changes in solvent accessibility produced upon binding most likely account for the change in the redox potential.

The effect of the environment on the heme centre is also observed from the HYSCORE measurements. In heme model compounds, the symmetry of the paramagnetic entity is preserved in such a way that molecular, electronic and magnetic axes keep a well-defined relationship. Particularly, a Z -axis perpendicular to the heme plane is common to these three frames (García-Rubio et al. 2003; Alonso et al. 2007). As a consequence, HYSCORE spectra of these model systems in the g_z position show only two dq peaks, provided that the two hyperfine splittings of the axial nitrogen nuclei are equivalent, as well as those of the four porphyrin nitrogen nuclei. On the other hand, when the symmetry of the paramagnetic entity environment is broken, as in the heme centre within some proteins, the relationship between molecular and magnetic axes disappears (Alonso et al. 2007). Then inequivalence between nitrogen hyperfine splittings can be detected in HYSCORE spectra, as it is here shown in Figure 5D.

PSII is a labile complex, and the lack of luminal extrinsic subunits, including Cc₅₅₀, is not unusual during purification experiments (Martinson et al. 1998; Nagao et al. 2007; Grouneva et al. 2011). This could be particularly true in *Phaeodactylum*, as this diatom is not disrupted by freeze/thawing cycles in an osmotically stabilized buffer, and pressure disruption is thus required. This contrasts with PSII purification in the diatom *C. gracilis*, for which freeze/thawing disruption allowed to obtain PSII particles containing most of the extrinsic luminal subunits (Nagao et al. 2007). These studies determined that diatoms have an extra extrinsic protein, Psb31, in addition to the other four subunits also present in red algae: PsbO, PsbU, PsbQ' and PsbV (Enami et al. 1998; Okumura et al. 2008; Nagao et al. 2010). Reconstitution experiments of PSII samples have suggested that both in red algae and diatoms the binding of PsbV (and PsbU) requires previous PsbO and PsbQ' binding and, in the case of diatoms, also the binding of Psb31, the last three proteins being able to bind directly to PSII intrinsic proteins (Enami et al. 1998, 2003; Nagao et al. 2010). In particular, in *C. gracilis* the presence of Psb31 alone is described to be able to rebind more than 50% of Cc₅₅₀ as compared with the whole collection of extrinsic proteins (Nagao et al. 2010). Interestingly, in cyanobacteria the PsbV subunit (Cc₅₅₀) is reported to bind directly to the PSII core, in a manner essentially independent of the other extrinsic proteins (Enami et al. 2003). The differences in the Cc₅₅₀ binding between cyanobacteria and red and diatoms eukaryotic algae (revised in Ifuku and Noguchi 2016) are intriguing, as the recent crystal structure of the *C. caldarium* (red alga) PSII has revealed an overall structure similar to the cyanobacterial PSII, which includes the position of Cc₅₅₀ in the complex (Ago et al. 2016).

Previous isolation of *Phaeodactylum* thylakoid-enriched membrane fractions lead to the lack of the five extrinsic subunits of PSII (PsbO, PsbU, PsbQ', Psb31 and PsbV (Grouneva et al. 2011). Here, thylakoid membrane samples containing 35–40 % of the total Cc₅₅₀ could be obtained by lowering the disruption pressure. This amount of membrane-bound cytochrome is in rough agreement with previous studies based in the EPR spectra of Cc₅₅₀ recorded in *T. elongatus* cells, which suggested the presence of a significant concentration of soluble Cc₅₅₀ in cells that could represent between 40–60% of the bound population (Kirilovsky et al. 2004). In our case, and as a control of other PSII extrinsic subunits, Psb31 was also monitored and detected in the membrane samples. However, the very low affinity of *Phaeodactylum* Cc₅₅₀ for PSII is demonstrated by the fact that Cc₅₅₀ is almost completely released along PSII purification through detergent solubilization of the isolated membrane fraction, and thus the protein mostly appears in the free (not-associated to PSII) gradient fraction (Figure 3). Remarkably, Psb31 remains bound to PSII, and thus the

release of Cc₅₅₀ seems to be –at least partially– specific. The fact that Cc₅₅₀ can be obtained as a soluble protein, not only in *Phaeodactylum* but also in other different organisms, could suggest the occurrence of unknown functions associated to the soluble form.

It is well known that iron availability limits growth of photosynthetic algae (Moore et al. 2002; Morrissey and Bowler 2012). Diatoms present a specific problem regarding the iron requirements of their photosynthetic apparatus, both because they have Cc₅₅₀ associated with PSII, and because lack the copper-protein plastocyanin replacing Cc₆ as an alternative donor to photosystem I (Bernal-Bayard et al. 2013, 2015). This extra iron requirement determines a lower adaptability of diatoms to the existing low levels of iron in large areas of the oceans. A down-regulation under iron limitation of several iron-containing proteins has been previously reported in coastal diatoms (Allen et al. 2008; Nunn et al 2013). This down-regulation includes ferredoxin (replaced by flavodoxin), PSI and some subunits of the *b₆f* complex (Allen et al. 2008; Morrissey and Bowler 2012; Nunn et al 2013). However, in *Phaeodactylum*, in particular, the D1 content and PSII concentration are basically unchanged under iron-deplete conditions as determined biochemically (McKay et al. 1997; Allen et al. 2008), whereas PSI and Cc₆ contents are significantly reduced to 30-40% from the values determined under iron-replete conditions (Allen et al. 2008; Roncel et al. 2016). Interestingly, this is also the case of Cc₅₅₀, for which a decrease of 45-50% in the protein content was determined under iron limitation (Figure 4A). In addition, changing iron availability in cultures acclimated to iron-replete or iron-deplete conditions promoted opposite effects in the Cc₅₅₀ content, *i.e.*: an increase when increasing the iron concentration in the media and a decrease when decreasing iron availability, the adaptation to the new conditions occurring in a time period of 6-8 days (Figure 4B). Actually, our results suggest a similar iron-regulation process for the two main luminal heme proteins, Cc₆ and Cc₅₅₀, and it is interesting to note that although the decrease in the protein content for the two cytochromes is different under iron limiting conditions, according to the Cc₆/Cc₅₅₀ ratio of ≈ 1.15 previously proposed, the final result is an equivalent concentration for both proteins.

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Supporting Information Available.

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TABLE 1. Comparison of EPR parameters of Cc₅₅₀ from *P. tricornutum* and other species

	g_z	g_y	g_x	Δ/λ	V/λ	V/Δ	pdb code
Soluble Cc₅₅₀							
<i>P. tricornutum</i>	3.00	2.24	1.44	3.17	1.71	0.54	-
<i>A. nidulans</i> ^a	2.98	2.24	1.46	3.22	1.75	0.54	-
<i>T. elongatus</i> ^a	2.97	2.24	1.49	3.35	1.80	0.54	1MZ4
<i>A. maxima</i> ^b	2.90	2.27	1.54	3.29	1.97	0.60	1F1F
<i>Synechocystis</i> 6803 ^{a,c}	2.87	2.28	1.57	3.32	2.06	0.62	1E29
PSII-bound Cc₅₅₀							
<i>T. elongatus</i> ^a	3.02	2.20	1.45	3.46	1.68	0.48	4V62, 2AXT
<i>Synechocystis</i> 6803 ^c	2.88	2.23	1.50	3.28	1.91	0.58	-

^a Values reported by Kerfeld et al. (2003); ^b Values reported by Sawaya et al. (2001); ^c Values reported by Vrettos et al. (2001).

FIGURE LEGENDS

Figure 1. Different purification and protein extraction procedures carried out during the characterization of Cc₅₅₀ from the diatom *Phaeodactylum tricorutum*. Asterisks indicate samples analyzed by MALDI-TOF in Figure 2. See text for further details.

Figure 2. (*Upper*) Molecular weight MS-analysis of different samples obtained during the purification of Cc₅₅₀ from *Phaeodactylum tricorutum*. (A) Cc₅₅₀ purified from the soluble cell extract; the peak on the left corresponds to the Cc₅₅₀ main peak at $z = 2$. (B) Cc₅₅₀ sample obtained from the salt-detergent washing of the membrane fraction. (*Lower*) Protein sequence of *Phaeodactylum* Cc₅₅₀ as translated from the *psbV* gene, and theoretical MW of the complete protein or different truncated forms.

Figure 3. (*Upper*) Western blot analysis of the different fractions acquired along the obtention of PSII-enriched samples from *Phaeodactylum tricorutum* as indicated in Figure 1 (M, molecular weight standard). Cc₅₅₀ and both the D1 core and the Psb31 extrinsic subunit of PSII were observed. For a comparative monitoring of each protein in the different fractions, in lines 1-4 equivalent sample volumes were loaded related to the initial volume of crude extract, whereas in lines 5-6 similar volumes directly extracted from the sucrose gradient bands were loaded. (*Lower*) Spectroscopic monitoring of cytochrome *b*₅₅₉ (Cb₅₅₉) of PSII (ascorbate *minus* ferricyanide, continuous line) and Cc₅₅₀ (dithionite *minus* ascorbate, dashed line) in: (5) the top of the gradient; (6) the lower green band in the sucrose gradient. PSII was monitored by the absorbance changes corresponding to cytochrome *b*₅₅₉.

Figure 4. (A) Cc₅₅₀ content in *Phaeodactylum tricorutum* cultures grown under iron-replete or iron-deplete conditions, as indicated, estimated by the specific redox differential absorbance changes (dithionite *minus* ascorbate). (*Inset*) Expanded spectra in the region of the Cc₅₅₀ α -band. (B) Variations in the Cc₅₅₀ content of cultures after changing iron availability. Cells growing in iron-replete (○) or iron-deficient (□) media, were resuspended in the same fresh medium; (■) cells growing in iron-deficient medium were resuspended in fresh iron-replete medium; (●) cells growing in iron-replete medium were resuspended in fresh iron-deficient medium. See the Experimental Procedures section for further information.

Figure 5. EPR spectra of Cc_{550} from *Phaeodactylum tricornutum*. (A) CW-EPR spectrum taken at $T = 25$ K. (B) Echo-detected EPR, $T = 6$ K, $\pi = 96$ ns. (C) Hole model. Energy levels of the t_{2g} orbitals in C_{2v} symmetry and definition of the parameters Δ and V . (D) HYSCORE performed at the magnetic field corresponding to g_z ($B = 230$ mT). $\tau = 96$ ns, $T = 8.5$ K. Double-quantum correlation peaks are indicated with arrows.

Figure 6. (A) Backbone model of Cc_{550} from *Phaeodactylum tricornutum* obtained using the program Phyre², with the crystal structures of Cc_{550} from the cyanobacteria *Thermosynechococcus elongatus* (pdb 1MZ4) and *Synechocystis* sp. PCC 6803 (pdb 1E29) as main templates. (B-E) Surface electrostatic potential distribution of the structural model of Cc_{550} from *Phaeodactylum* either in (B) the complete and (C) truncated forms, (D) *Synechocystis* 6803 and (E) the red alga *Cyanidium caldarium* (pdb 4YUU). The view displays the heme groups in the same orientation, showing in front the cofactor exposed area and in the top the protein C-terminal part. Simulations of surface electrostatic potential distribution were performed using the Swiss-Pdb Viewer Program assuming an ionic strength of 500 mM at pH 7.0. Positively and negatively charged regions are depicted in blue and red, respectively.

Figure 7. Detail of surface electrostatic potential distribution around the heme group of Cc_{550} from: (A) *Phaeodactylum tricornutum* (model shown in Figure 6C), (B) *Thermosynechococcus elongatus*, (C) *Arthrospira maxima* (pdb 1F1C) and (D) *Synechocystis* sp. PCC 6803. The view shows in front the heme group exposed area. Positively and negatively charged regions are depicted in blue and red, respectively. The same view of the entrance to the heme pocket is displayed for *T. elongatus*, *Synechocystis* 6803 and *A. maxima* structures and for the *Phaeodactylum* model. See Figure 6 for further details.

Supplementary Material.

Figure S1. Absorption spectra of Cc₅₅₀ (10 μM) from *Phaeodactylum tricornutum* in its native oxidized form (continuous line) and after reduction with dithionite (dashed line). (*Inset*) Reductive potentiometric redox titration of purified Cc₅₅₀ (10 μM) in potassium phosphate 50 mM, pH 7, buffer. Continuous line corresponds to the theoretical fit according to the Nernst equation and n=1. See the Experimental Procedures section for further information.

Figure S2. BrCN cleavage and peptide analysis of Cc₅₅₀ from *Phaeodactylum tricornutum*. (*Upper*) Theoretical protein sequence as deduced from the translation of the *psbV* gene. Methionine targets for BrCN are underlined. (*Middle*) Molecular weight MS-analysis of the different peptides obtained during the BrCN digestion of Cc₅₅₀. Arrows indicate the peptides fitting the expected results from the digestion of the truncated Cc₅₅₀. (*Lower*) Expected main peptides from the Cc₅₅₀ cleavage by BrCN in methionine positions, either in the theoretical protein (1-3) or the truncated form (1,2,4,5). Peptide 3 was not detected.

Figure S3. (*Left*) Cc₅₅₀ and (*right*) Cc₆ content in the soluble fraction (continuous line) of *Phaeodactylum tricornutum* disrupted cells, and in samples obtained after treating the membrane fractions with NaCl and detergent (dashed line), as estimated from the differential absorbance spectra (reduced *minus* oxidized). See the Experimental Procedures section for further information.

Figure S4. Sequence alignment of Cc₅₅₀ from *Phaeodactylum tricornutum*, *Termostynechococcus elongatus*, *Arthrospira maxima* and *Synechocystis* sp. PCC 6803. Red arrows point to the axial heme histidine ligands.

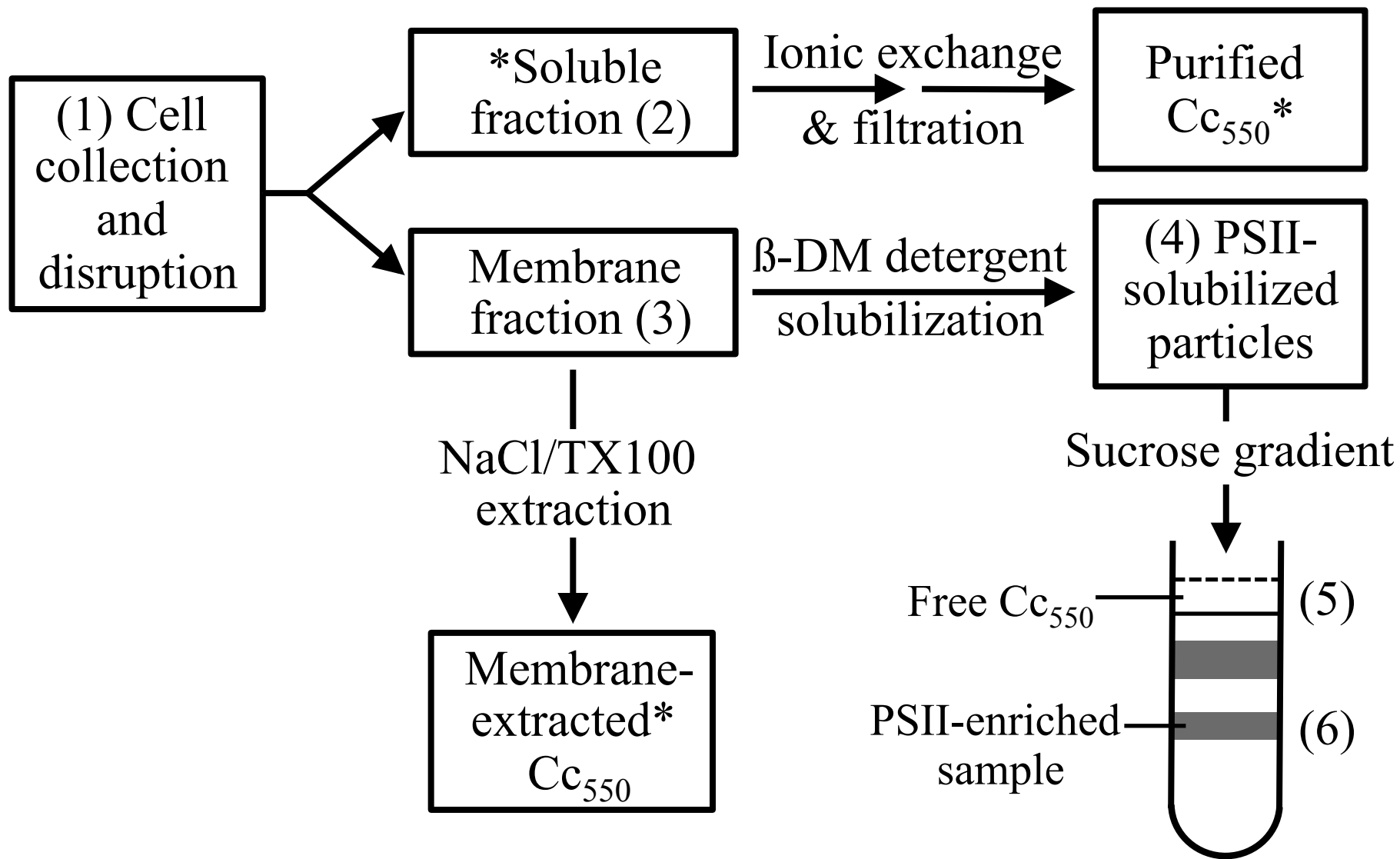
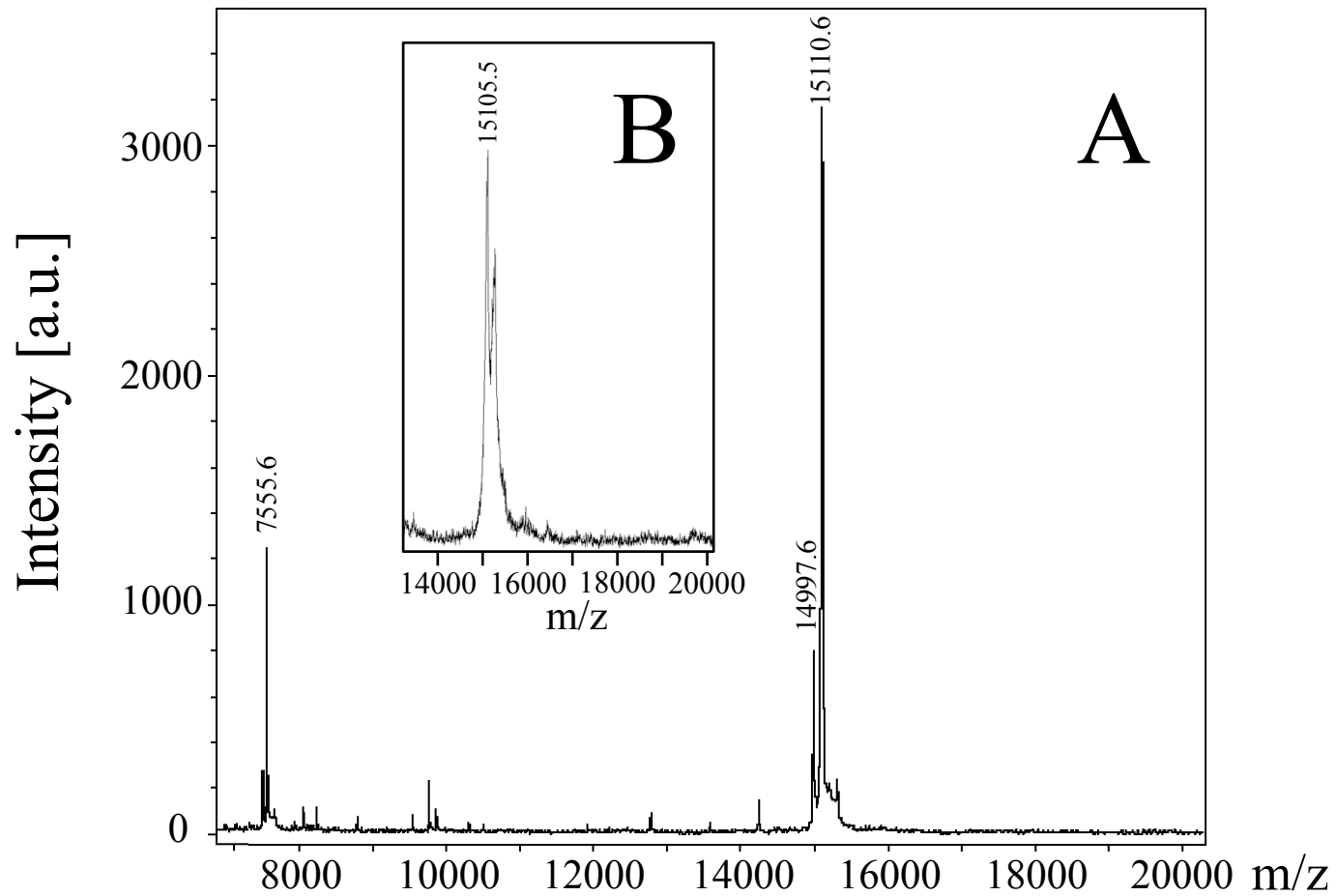


Figure 1

Figure 2



IDLDEATRTV VVDSSGKTIV LTPEQVKRGK RLFNATCGAC
HVGGVTKTNP NVGLDPEALS LATPRRDNIA GLVDFLKNPT
TYDGLIESIAE VHPSIKSADI YPRMRSVTDE DLTAMAGHIL
LQPKIVTEKW GGGKIYY MW = 15,438; *psbV* gene sequence
LQPKIVTEKW GGGKI MW = 15,111; truncated 2-C-terminal
LQPKIVTEKW GGGK MW = 14,998; truncated 3-C-terminal

Figure 3

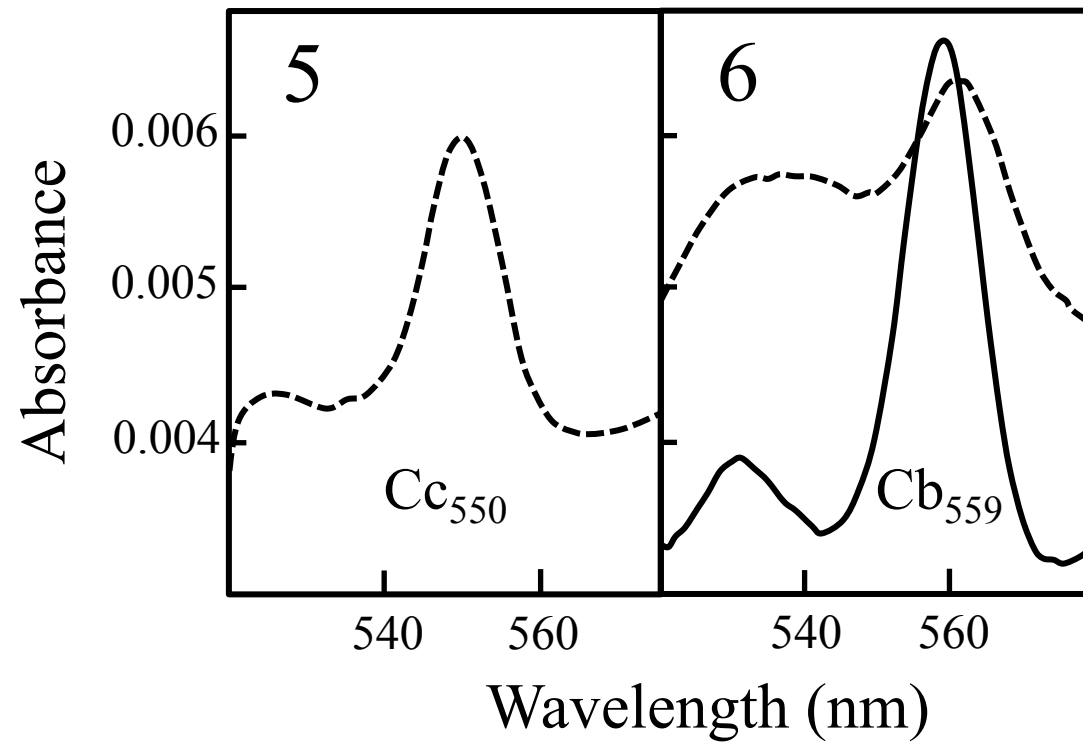
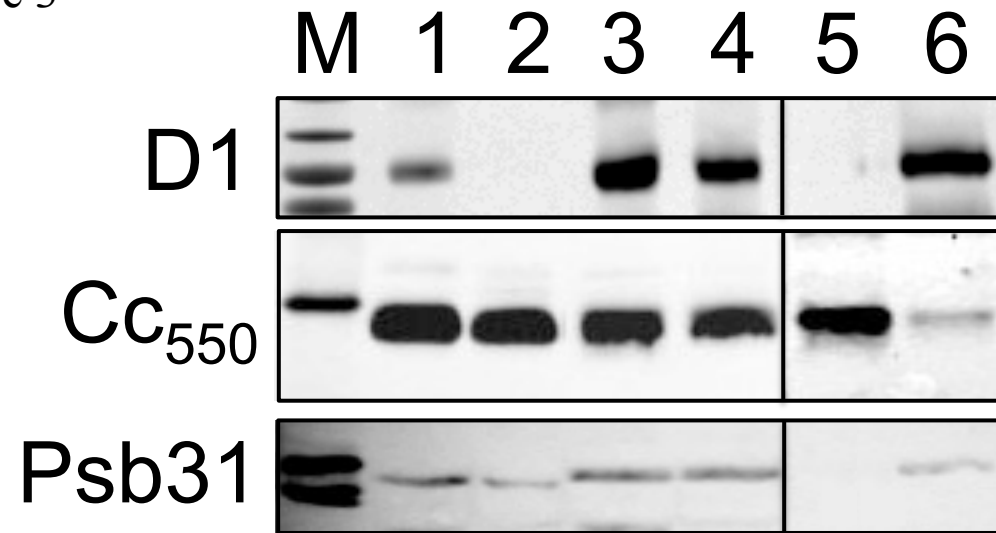
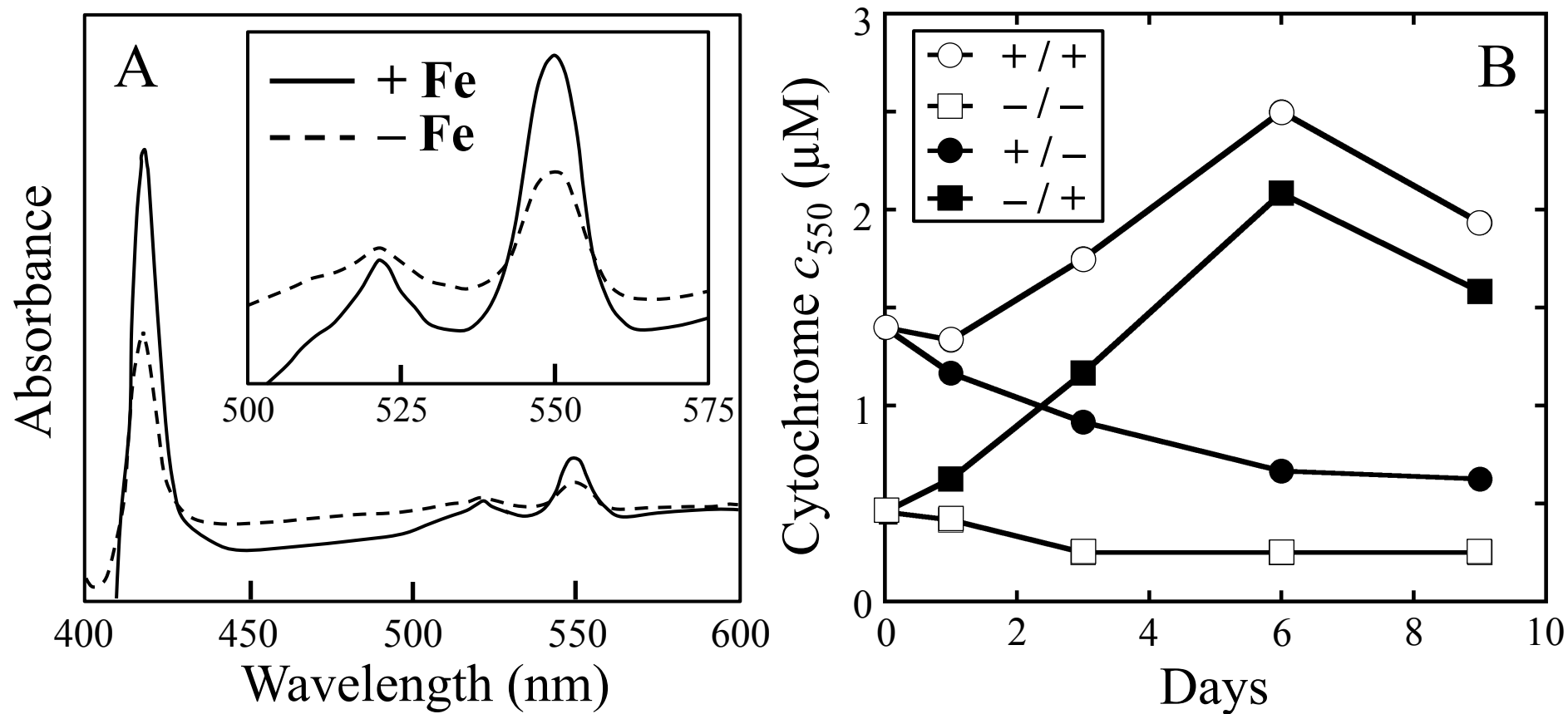


Figure 4



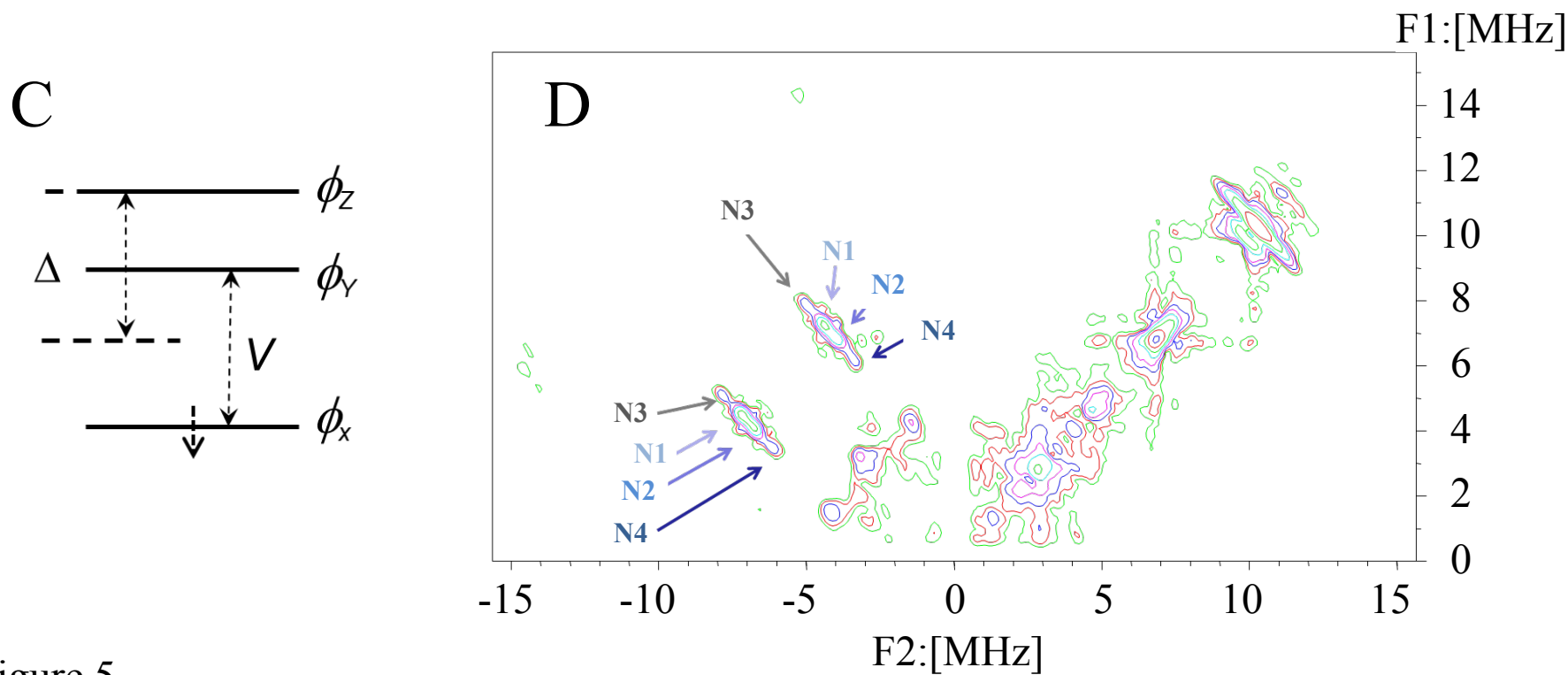
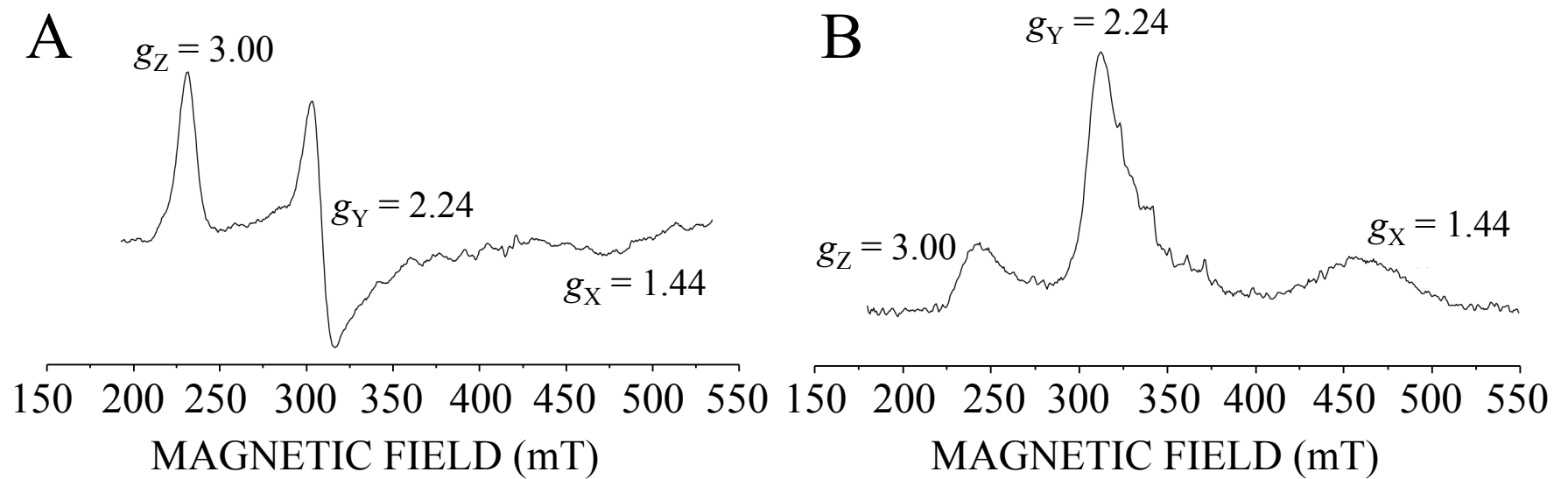


Figure 5

Figure 6

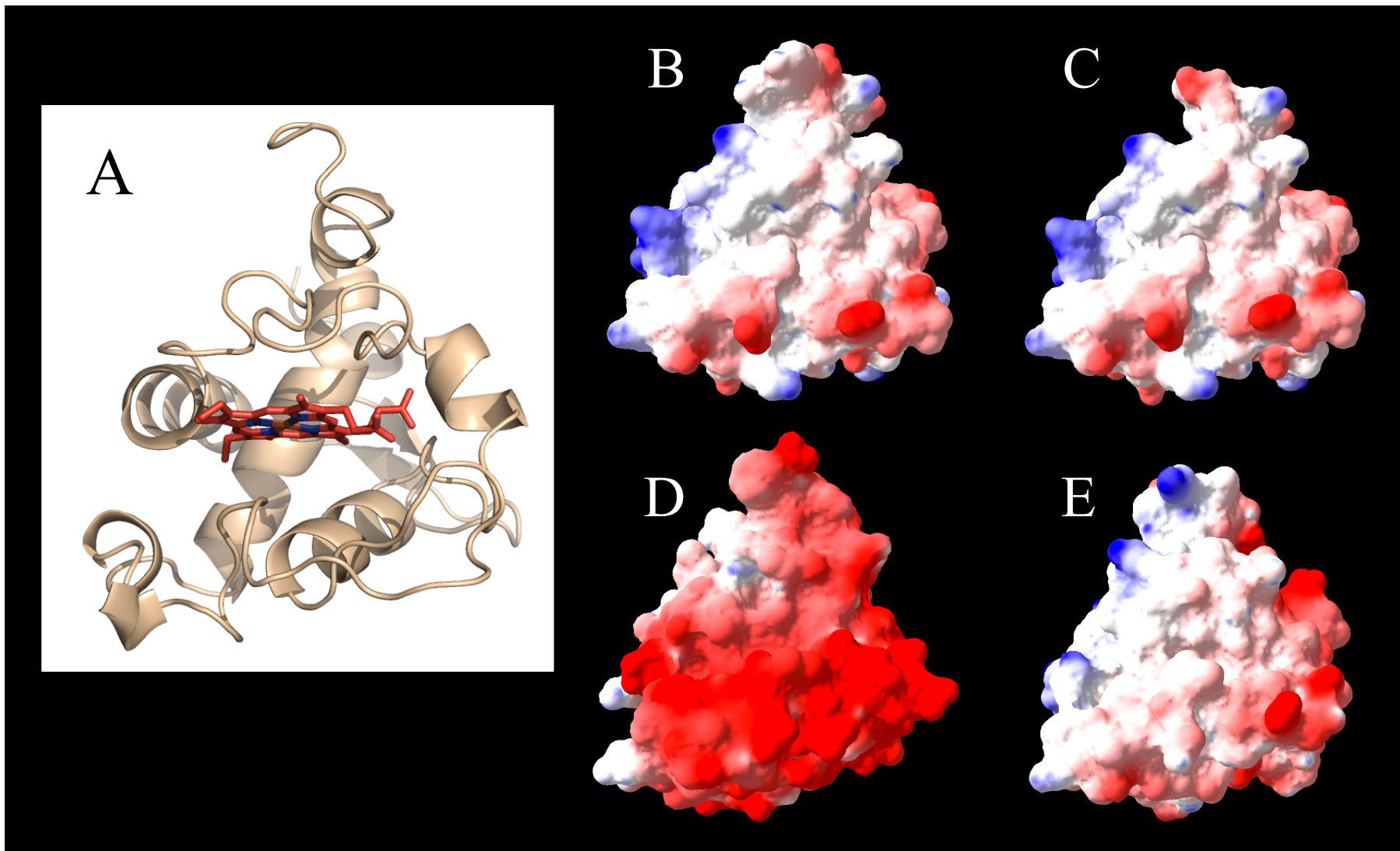
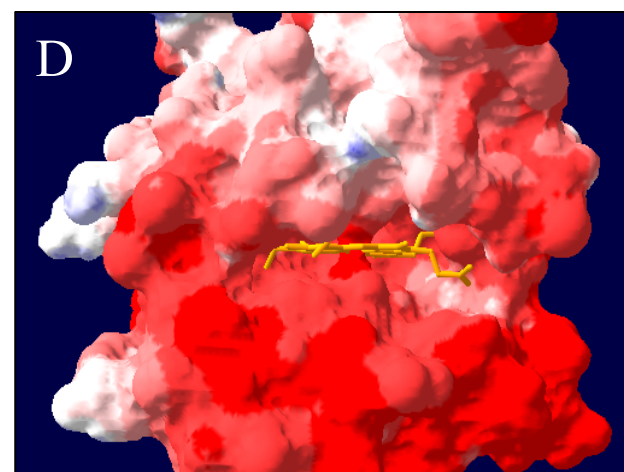
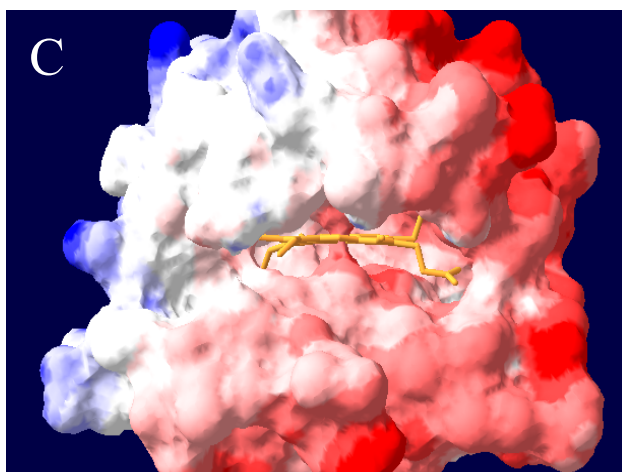
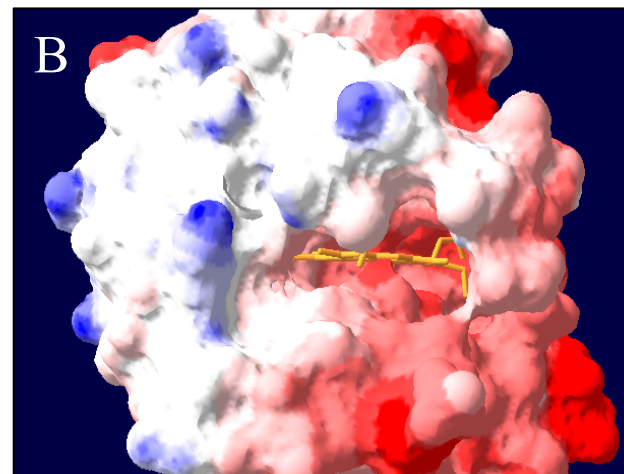
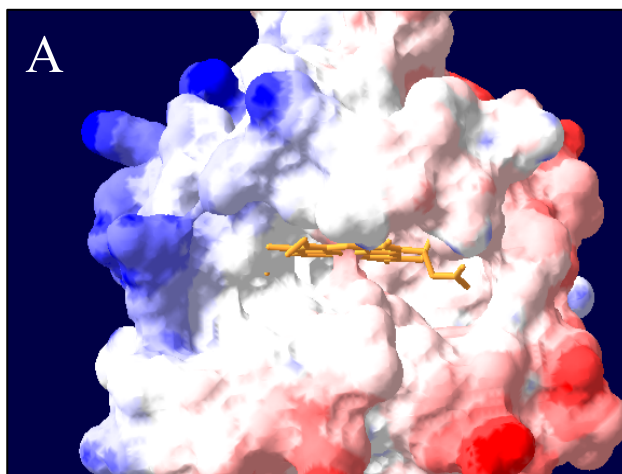


Figure 7



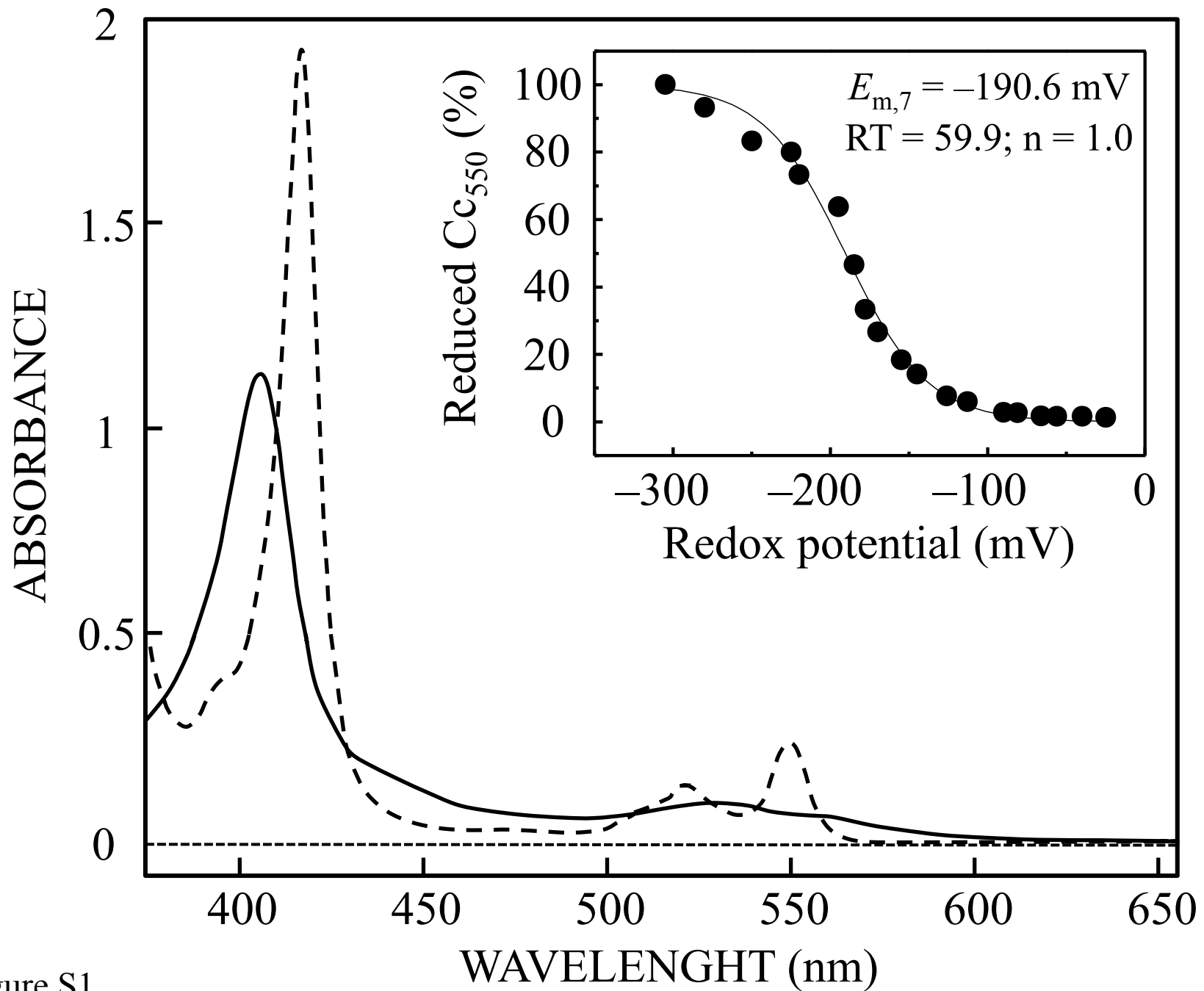
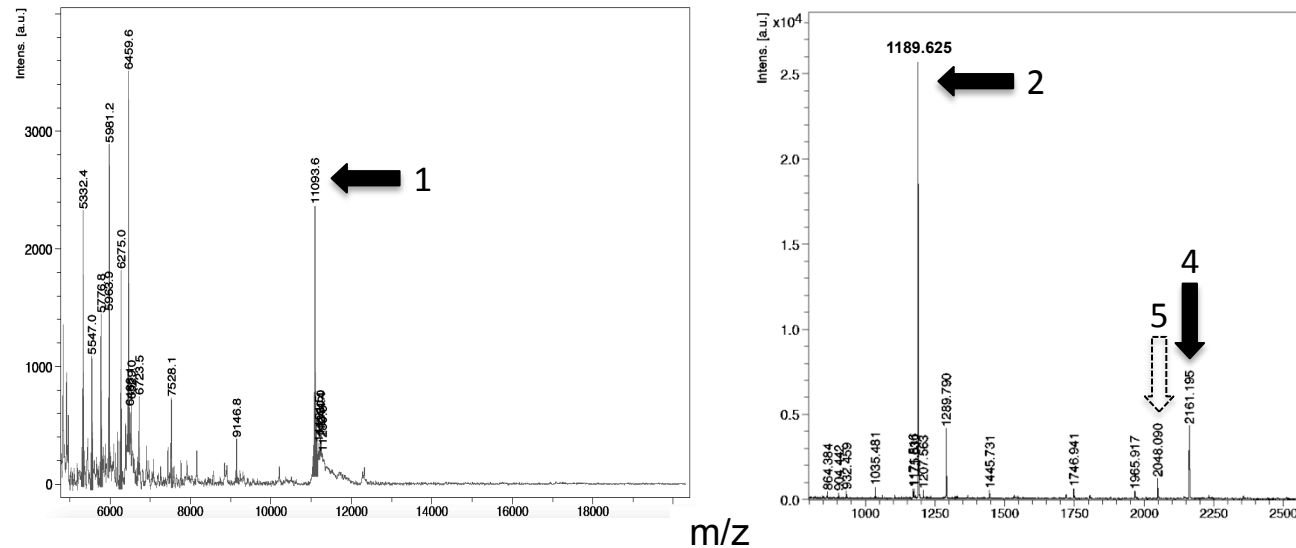


Figure S1

Figure S2

IDLDEATRTV VVDSSGKTIV LTPEQVKRGK RLFNATCGAC HVGGVTKTNP
NVGLDPEALS LATPRRDNIA GLVDFLKNPT TYDGLIESIAE VHPSIKSADI
YPRMRSVTDE DLTAMAGHIL LQPKIVTEKW GGGKIYY



1. IDLDEATRTV VVDSSGKTIV LTPEQVKRGK RLFNATCGAC HVGGVTKTNP
NVGLDPEALS LATPRRDNIA GLVDFLKNPT TYDGLIESIAE VHPSIKSADI
YPRM

2. RSVTDE DLTAM

3. AGHIL LQPKIVTEKW GGGKIYY

4. AGHIL LQPKIVTEKW GGGKI

5. AGHIL LQPKIVTEKW GGGK

Figure S3

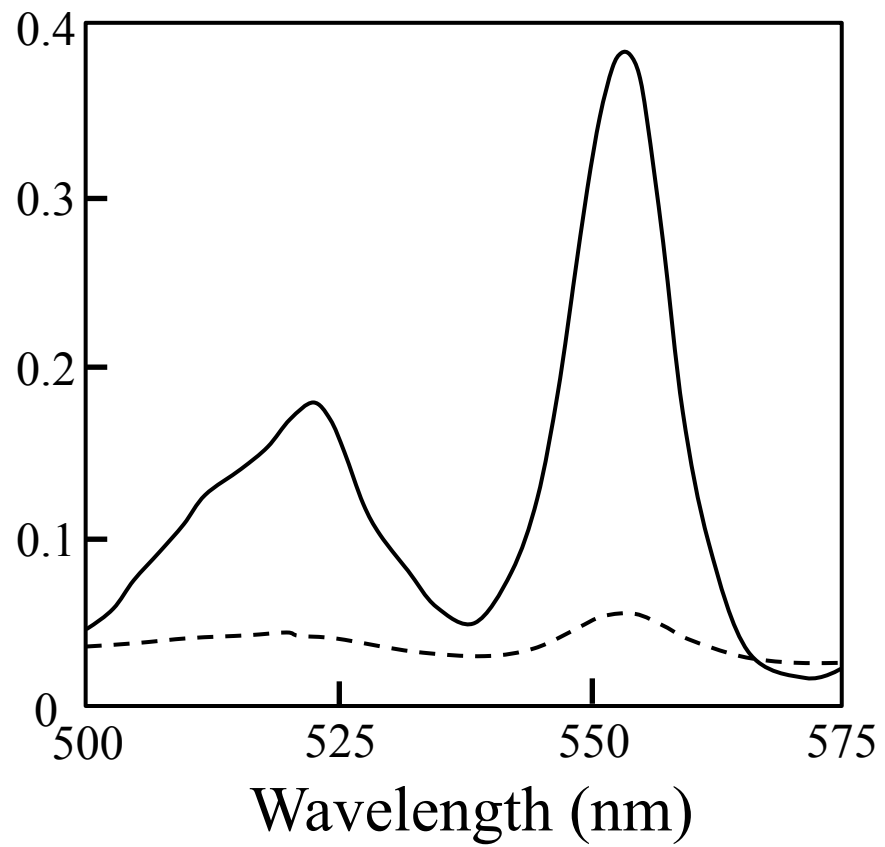
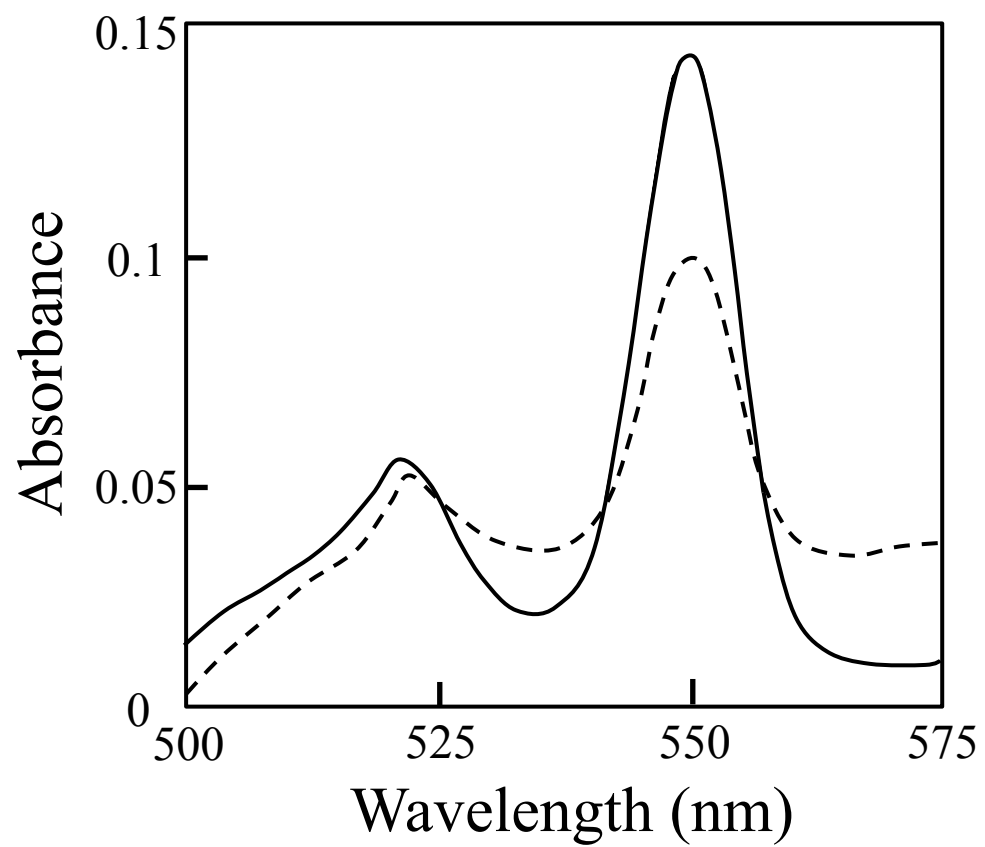


Figure S4

